"Exploring potentials of *Beta vulgaris* along with dietary supplements in the treatment of Alzheimer's disease."

SUMMARY FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY (Ph.D.)

in

Pharmaceutical Sciences

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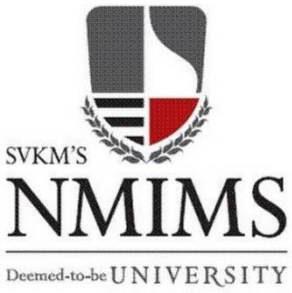

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1. Introduction:

Alzheimer's disease (AD) is a multifaceted and severe CNS neurodegenerative condition. It is brought on by significant cognitive decline, persistent memory loss, and loss of orientation, reasoning, and functioning abilities (Fang et al., 2013; Hardy and Selkoe, 2002). One of the leading causes of impairment and dependence among older people worldwide, dementia is presently the seventh most significant cause of mortality. In England and Wales, Covid-19 was the most significant cause of death in 2020 and 2021, but dementia and AD, which accounted for 11.4% of all deaths in 2022 and resulted in 65,967 fatalities, became the leading cause of death in 2022 ("Dementia and Alzheimer's leading cause of death in England and Wales _ Society _ The Guardian," n.d.).

AD onset is closely related to neuroinflammation caused by microglia. Activated microglia in the chronic stage of neuroinflammation can accelerate brain inflammation. Ongoing neuroinflammation leads to Aβ plaque deposition and the failure of clearance. The blood-brain barrier (BBB) disruption, BBB transporter impairment, and a decline in the Aβ degrading enzyme may be the causes of Aβ clearance failure due to neuroinflammation (McGeer et al., 2000; Munoz and Ammit, 2010; Sastre et al., 2006). Low-density lipoprotein receptor-related protein-1(LRP-1) overexpression and receptor for advanced glycation end products (RAGE) level downregulation might occur in BBB transporter malfunction. Neprilysin (NEP), insulin-degrading enzyme (IDE), endothelin-converting enzyme 1 (ECE1), angiotensin-converting enzyme (ACE), and endothelin-converting enzyme 2 (ECE2) all play essential roles in the breakdown of Aβ. The accumulated Aβ may have an unfavorable effect on synaptic function by downregulating CREB and decreasing glucose absorption. In addition, it may result in neurodegeneration and a reduction in cognitive impairment; this pathophysiology paves the way for dementia and AD (An et al., 2018; Cai et al., 2014; Teich and Arancio, 2012).

An inflammasome, a multiprotein complex, accelerates microglial inflammation via the IL-1β synthesis process. The NLRP3 inflammasome, which consists of the caspase-1 protease, the NLRP3 sensor, and the ASC adaptor, is one of the inflammasomes that has been the subject of the most research. It is predominantly expressed in microglia and more directly linked to the pathophysiology of AD, acting as a mediator of microglial recruitment to foreign Aβ plaques and

detecting Aβ. Today, evidence connects the NLRP3 inflammasome to several diseases, including AD (Cai et al., 2022; Chen et al., 2021).

Many pathogenic neuroinflammatory reactions are characterized by an excessive release of the pro-inflammatory cytokine IL-1β, which leads to glutamate release via post-synaptic NMDA receptor activation and synaptic loss. It is widely acknowledged that IL-1β significantly impacts CNS diseases like AD. The inflammasome is increasingly essential for regulating the maturation of IL-1β (Bernier, 2012; Mishra et al., 2012; Rosa et al., 2021). Therefore, it is vital to shed light on microglia-induced neuroinflammation. Targeting NLRP3-driven neuroinflammation triggered by microglia could be beneficial (Sarlus and Heneka, 2017).

Dietary supplements are substances that can be taken to enhance the nutritional value of your diet or lower the risk of developing health problems. These supplements include tablets, capsules, liquids, powders, and gel capsules ("What are dietary supplements?," 2002). An unhealthy diet causes neurodegenerative disorders and impaired cognitive function. Over time, adopting a balanced diet would prevent and control neurological disorders and brain aging (Naureen et al., 2022). Many Americans take dietary supplements. Recent polls showed that over 50% of people in the US use them (Nesheim, 1998).

Certain foods and herbs have been found to strengthen the brain. *Ginkgo biloba* extract made from *Ginkgo biloba* is commonly used for AD patients. It reduces blood viscosity, and the brain can receive more oxygen, improving brain function. Huperzine-A, a compound produced from the Chinese moss *Huperzia strata*, is believed to block a brain enzyme that affects acetylcholine (Qian and Ke, 2014; Xie et al., 2022)

Some dietary supplements may also enhance brain function. DMAE, or 2-dimethylaminoethanol, may affect acetylcholine levels in the brain. Acetyl-L-carnitine has been found to help maintain brain cells and aid those with minor memory loss or early-stage Alzheimer's disease. Omega-3 fatty acid, specifically docosahexaenoic acid (DHA) found in fish oils, is vital for a growing baby's brain development and may improve brain function by improving communication between brain cells (Pennisi et al., 2020; Shipkowski et al., 2019). Maintaining brain health requires the inclusion of dietary supplements and herbs hence investigating the role of nutritional supplements and herbs in neuroinflammation-induced AD is crucial.

Betanin (BET), one of the betalains (betanidin 5-O-D-glucoside, red), makes up 75–95% of the total betalain and is derived from *Beta vulgaris* (Family Caryophyllales) and is used as a health food. It shows tremendous promise in treating inflammatory and oxidative stress-related diseases, including cancer, arthritic conditions, and liver disease. *In vitro* and many *in vivo* animal models, research has revealed that betalains have potent antioxidant, radioprotective, and anti-inflammatory effects (Kaur et al., 2018).

Fresh coconut fruits of the species *Cocos nucifera* are used to produce virgin coconut oil. The VCO diet or supplementation avoided rodent nephrotoxicity from gentamicin, cadmium, and neurotoxicity. It is an excellent adjuvant in treating oxidative damage and inflammation brought on by chemotherapy. Vascular endothelial function is improved with a VCO supplement (30 mL/day). VCO supplementation has been helpful with other drugs (Famurewa et al., 2020, 2018b, 2018a; Shehata et al., 2020).

VCO in the body after intake is converted into Medium chain triglycerides (MCT). Then it forms medium-chain fatty acid (MCFA). A more prominent MCFA is lauric acid. It provides energy via ketone bodies and is a substitute energy source for the brain in altered glucose metabolism. This condition is a hallmark of AD.

To tackle the intricate nature of AD, it may prove beneficial to administer a combination of medications that can effectively target numerous components simultaneously.

2. Hypothesis

When the microglia are activated, they release the cytokine IL-1β, which can accelerate neuroinflammation. Mitochondrial dysfunction can lead to the activation of NLRP3 inflammasomes, which produce IL-1β. It is essential to control neuroinflammation to prevent the progression of the disease. Recent research has revealed that betanin, a natural compound found in beets, has anti-inflammatory and antioxidant properties that could potentially be used to control neuroinflammation.

In glucose hypometabolism conditions, ATP deficit can cause neuronal damage; therefore, ketone bodies obtained from VCO via MCTs can serve as an alternative energy source to produce ATP. This approach can prevent further neuronal damage while meeting the body’s ATP needs. Combining Betanin with VCO is a logical choice to control neuroprotection and

neuroinflammation concurrently. This combination primarily inhibits NLRP3 inflammasome activation, preventing the production of IL-1β and ultimately halting AD advancement (Thawkar and Kaur, 2018).

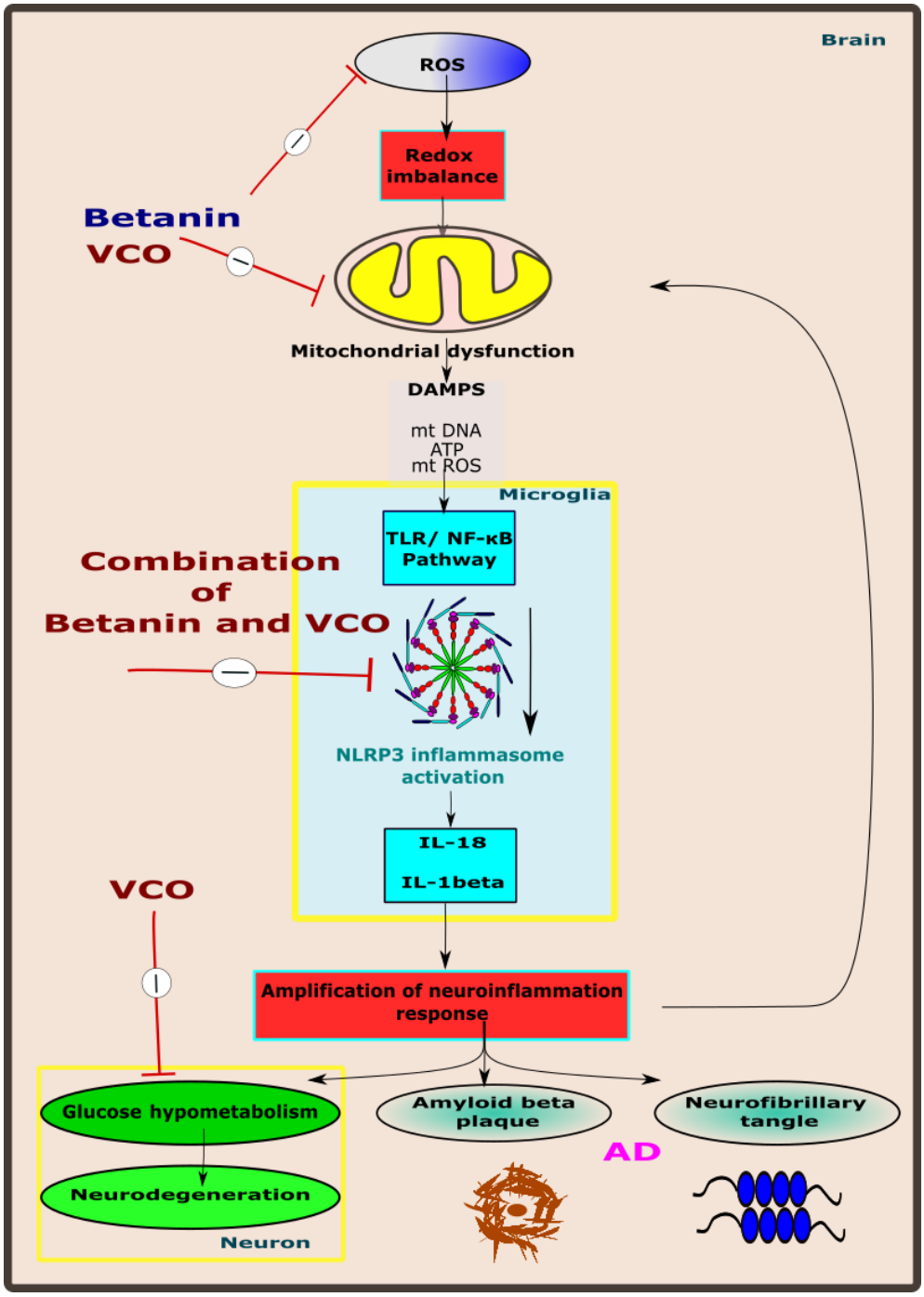

Fig 1: Selective targets of combination during neuroinflammation.

3. Objectives:

1. Characterization of *Beta vulgaris* spray-dried powder.
2. Characterization of virgin coconut oil
3. To evaluate the safety of *Beta vulgaris* and Virgin coconut oil as per OECD guidelines
4. To evaluate the potential neuroprotective role of *Beta vulgaris* by using a Zebrafish model.
    a. Evaluation of behavioral Studies
    b. Determination of Antioxidant and AChE activities in the whole brain
5. To study the effect of *Beta vulgaris* and virgin coconut oil in Aluminum chloride (AlCl3) induced behavioral deficits in Wistar rats.
    a. Evaluation of behavioral Studies
    b. Estimation of potential biomarkers
    c. Determination of Antioxidant and AChE activities in the whole brain
    d. Histopathology of the isolated brain from the rat
6. To study the effect of *Beta vulgaris* and virgin coconut oil in lipopolysaccharide-induced Alzheimer's disease in C57BL mice.
    a. Evaluation of Behavioral Studies such as Morris water maze, Elevated plus maze test, and locomotor activity.
    b. Evaluation of oxidative stress parameters and AChE activities in the whole brain.
    c. Estimation of NLRP3 and IL-1β level by ELISA method.
    d. Histopathology of the isolated brain from the mice.

4. Characterization of Betanin by LCMS

4.1. HPLC Method:

The solvent system comprised solvent A, a mixture of water/formic acid (99:1, v/v), and solvent B, acetonitrile/formic acid (99:1, v/v). The column used Reliasil 2 5µm C18 250x 4.6mm S/N 21704192. The HPLC program: a linear gradient from 3% to 14% B in 17 min; Flow rate 0.6 ml/min; Injection volume of 30 µl UV–Vis detection at 484 and 535 nm (Otálora et al., 2015).

4.2. Mass spectroscopy

The MS parameters were as follows: positive and negative mode; CDL temperature 200 °C; heating block at 450 °C; detector voltage, 2.00 kV; flow nebulizing gas (N2), 3 L/min; and scan range m/z, 50–1000 u. The LC-MS Solution software (Shimadzu, Tokyo, Japan) was used for data collection and analysis

5. Characterization of virgin coconut oil by GCMS

The fatty acid contents of the VCO have been evaluated by the Gas Chromatography Varian-CP-3800 (Varian, Inc., CA, USA) equipped with a flame ionization detector (FID) and a CP-Sil-88 column with a length of 100m and a split ratio of 10:1 were used. The standards identified fatty acids by comparing the resultant picks and related retention time (Rt) (Mirzaei et al., 2018).

6. Acute toxicity study of the combination

The acute toxicity studies of the combination were performed according to Organization of Economic Corporation Development Guidelines No. 423 using SD female rats. Three rats were given betanin (2000 mg/kg) and VCO (10,000 mg/kg) orally, and any instances of death or severe abnormalities were recorded over a 14-day monitoring period after the administration of the doses. Based on the acute toxicity study, 100 and 200 mg/kg oral doses for betanin and 5 & 10 g/kg of VCO were selected for the study (OECD, 2002).

7. Betanin alleviates scopolamine-induced cognitive impairment by restoring cholinergic function, boosting brain antioxidative status, and increasing BDNF levels in the zebrafish model.

In the last decade, the zebrafish (Danio rerio), a small tropical freshwater fish, has become increasingly important as a model for studying behavioral pharmacology. This species provides a distinct way to explore the correlation between drug effects and behavior, serving as a valuable alternative to traditional mammalian models and in vitro studies (Bailey et al., 2015). Hence current study is undertaken to screen the effect of betanin on zebrafish for memory-improving activity (Thawkar and Kaur, 2023).

7.1. Materials and Methods

7.1.1. Drugs

Betanin (Red Beet extracts diluted with Dextrin, CAS 7659-95-2, TCI chemicals), Scopolamine hydrobromide trihydrate (C17H21NO4·HBr·3H2O, CAS 6533-68-2, Sigma-Aldrich Corporation, St. Louis, MO, USA.), Donepezil hydrochloride was procured as a gift sample from Micro Labs Limited, Mumbai, India: Fish Brain-derived neurotrophic factor, BDNF GENLISA™ ELISA(Krishgen Biosystems, India).

7.1.2. Animals

A total of 60 adult zebrafish were procured, belonging to the short-fin strain, comprising both male and female specimens, within the age range of four to five months, from a local aquarium store. These zebrafish were accommodated in 300mm x 200mm x 300mm aquariums and were provided with continuous aeration. The fish tank was subjected to a controlled environment where 10 hours of darkness followed by 14 hours of light was maintained each day, with the lighting system turning on promptly at 8:00 am. The tank adhered to a standard of five animals per liter of fluid. The fish was fed a commercial flake meal sourced from Hello Pets based in Mumbai, India, and provided twice daily. The water in the tank undergoes a complete replacement every three days to ensure optimal hygiene and cleanliness.

Water quality parameters were monitored daily by Aquaculture Chemtest Kit and kept in the following ranges: pH (7.5-8), Chlorides 30-40 ppm, Total hardness (75-80 ppm as CaCO3), Carbon dioxide (5-7 ppm as CO2), Nitrite (0.02-0.03 ppm), Ammonia (< 0.5 ppm), Alkalinity (80-85 ppm as CaCO3), Dissolved oxygen (5.2-6.3 ppm as O2), and temperature (28±0.5 °C). The Institutional Animal Ethics Committee permitted the study protocol (approval no. CPCSEA/IAEC/P-18/2019 dated March 22, 2019), which was formed following the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

7.1.3. Toxicity studies: as per the OECD. guideline 203

In a 1-liter aquarium, betanin was diluted in concentrations (50,100,200,500,700, and 1000 mg/L). Ten animals for each concentration remained in contact with the solution for 96 hours. During this

period, behavioral changes and mortality were observed (da Silva Aleluia et al., 2018; OECD Guidelines and Development Economic Cooperation and, 1992).

7.1.4. Experimental design

In the experiment, all animals were separated into six groups: a control group, a scopolamine group (100 µM), and three betanin (BET) treatment groups (50 mg/L, 100 mg/L, and 200 mg/L), as well as a positive control group with donepezil(DP) (10 mg/L). The drug solution was prepared in the millipore water in the dosing tank, and fish were exposed for 1 hour every day between 12 pm to 3 pm. for up to 8 days (Holcombe et al., 2014). Fish were placed into a glass beaker (500 mL) with scopolamine (100µM) mixed in water and left for 1 hour before the training session. The control fish were placed in a glass of water and subjected to similar procedures. The fish were retrieved after the memory test for brain dissection and biochemical activity.

7.1.5. Behavioral Assessment

In all the tests, a Logitech HD Webcam C525 camera was used to record fish behavior videos, then analyzed using ANY-maze® software (Stoelting Co, USA.)

7.1.5.1. Novel tank test (N.T.T.)

NTT is used to study anxiety in fish. The tank measures 23.9 cm at the bottom, 28.9 cm at the top, 15.1 cm high, and 15.9 cm along the sloping side. Observations were recorded for 6 minutes. The following parameters were analyzed: a) total distance traveled (mm), b) The distance top/bottom ratio, c) The mean speed of zebrafish, d) Time spent in the top/bottom ratio, and time in the top zone of the tank (Capatina et al., 2020b; Valu et al., 2021).

7.1.5.2. Y-maze task

The Y-maze test is used to study the spatial memory test. Fish were examined in a Y maze tank with three arms measuring 25 x 8 x 15 cm (length, breadth, and height). The following parameter was analyzed: a) The absolute turn angle, b) time spent in each arm, c) Percentages preferences d) The total distance traveled by zebrafish in the tank (Boiangiu et al., 2021; Brinza et al., 2020; Capatina et al., 2020a; Cognato et al., 2012)**.**

7.1.6. Biochemical Parameters Assay

7.1.6.1. Collection of Brain Tissues

Fish were cryo-anesthetized, decapitated, and entire zebrafish brains were taken at the end of the behavioral experiments. A probe homogenizer (Polytron PT 2500E, Kinematica, Switzerland) was used to homogenize 100 mg of tissue in 0.5 ml phosphate buffer (0.1 M, pH 7.4). Different fractions of tissue homogenates were produced and processed to yield post-nuclear and post-mitochondrial fractions.

7.1.6.2. Determination of acetylcholinesterase activity

The Amplite^{TM} Colorimetric Acetylcholinesterase assay Kit was used to measure AChE activity according to the manufacturer's instructions. The reaction is initiated with the presence of the substrate acetylthiocholine, and the hydrolysis and production of the DTNB dianion were measured at 412 nm within 10 minutes using a microplate reader (ELLMAN et al., 1961; Richetti et al., 2011).

7.1.6.3. Determination of the oxidative stress parameters

Malondialdehyde (MDA) and reduced glutathione (GSH) assay were measured in the whole homogenate (Ellman, 1959; Ohkawa et al., 1979a). In addition, to extract post-nuclear supernatant (PNS), one fraction was centrifuged at 2,500 rpm for 20 minutes at 4°C. The activity of catalase was determined using this supernatant (Lück, 1965). Also, a superoxide dismutase (SOD) test was performed with a post-mitochondrial supernatant (PMS). The PMS was obtained by centrifuging homogenate at 10,000 rpm for 20 min at 4°C(Suryavanshi et al., 2021).

7.1.6.3.1. Determination of the MDA (malonyl dialdehyde) assay

The brain homogenate is used for this assay. The reaction mixture consists of 100µl of sample/standard, 8.1% w/v Sodium lauryl sulfate,20% v/v glacial acetic acid, 0.8% w/v thiobarbituric acid, and distilled water. Then the reaction mixture was incubated at 100^0C for 1 hour. After that, it was centrifuged at 10000g for 10 min. Measured absorbance of the supernatant at 532nm. The result was expressed as the amount of MDA formed per tissue weight as nmol of MDA/mg of protein.

7.1.6.3.2. Determination of GSH (reduced glutathione) assay

The brain homogenate was used for this assay.300µl of sample/standard was mixed with 300µl of 10% trichloroacetic acid. Then, the reaction mixture was kept for one hour at 4oC. The supernatant was collected after the centrifugation of the mixture at 12000g for 20 min.100 µl of supernatant was mixed with 0.2M phosphate buffer(pH-7) and DTNB, and measured the absorbance at 412nm. The result was expressed as (µmol of GSH/mg of protein).

7.1.6.3.3. Determination of the SOD Activity

The post-mitochondrial fraction was used for this assay.50µl supernatant was added to the reaction mixture of 50mM sodium carbonate (pH-10, containing 0.1mM EDTA),96µM nitroblue tetrazolium,0.02% triton, and 20 mM hydroxylamine HCL and measured the absorbance at 560nm for 2 min at the interval of 30 seconds. One unit of SOD is defined as the concentration of enzyme that gives half-maximal inhibition of NBT reduction. The enzyme activity was reported in units/mg of protein (Paoletti et al., 1986a).

7.1.6.3.4. Determination of the CAT Activity

The post-nuclear supernatant was used for this assay. The reaction mixture of 0.2M phosphate buffer (pH-7) and 25 µl sample mix well and kept for 30 seconds. Measured decrease in optical density for 2 min at 1min intervals at 240nm. Then, a 25 µl sample was added to hydrogen peroxide-containing 0.2M phosphate buffer (pH-7). A similar procedure was performed to determine optical density. Enzyme activity was measured using the molar extinction coefficient of hydrogen peroxide. The results were expressed as a micromole of H2O2 decomposed/min/mg protein.

7.1.6.4. Brain-Derived Neurotrophic Factor (BDNF)

Per the manufacturer's instructions, the BDNF level was determined using a commercially available GENELISA^{TM} Fish BDNF ELISA kit (Krishgen Biosystems, India). Absorbance was read at 450nm within 10-15 min. The results are as picograms (pg) of cytokine/mg of tissue.

7.1.6.5. Protein determination

The Bradford method measured the protein for the biochemical studies by considering bovine serum albumin as the standard. It is performed using a protein estimation kit. (HI Media Laboratories, India) (Bradford, 1976; Kruger, 1994)

7.2. Statistics

All values are expressed as mean ± SEM (n = 10). Statistical analyses were conducted using GraphPad Prism 8 software for Windows (GraphPad Software, San Diego, CA, USA). First, the normal distribution of the data was confirmed by Kolmogorov-Smirnov and Levene tests for behavioral and Shapiro-Wilk tests for biochemical parameters. Next, the differences between the groups were analyzed with a one-way analysis of variance, followed by Dunnett's multiple comparisons tests. Statistical significance was considered at $p < 0.05$.

8. The effects of Betanin and Virgin Coconut oil in aluminum chloride triggered an Alzheimer's disease model in rats via the NLRP3 inflammasome pathway.

8.1. Animals:

Male Albino Wistar rats weighing between 180-220g were sourced from the National Institute of Biosciences in Pune, India, after a week of acclimatization. The rats were maintained under optimal environmental conditions, including a 12/12 hr light/dark cycle, a room temperature of 25 ± 2◦C, and a relative humidity of 40-60%. Throughout the experiment, the rats were provided with ad libitum access to water and a standard pellet diet. The study protocol was approved through Institutional Animal Ethics Committee (approval no. CPCSEA/IAEC/P-24/2018, dated 6th September 2018).

8.2. Experimental Design

The seventy-two male albino Wistar rats (180–220 g) were randomly selected based on their body weight in nine groups of 8 animals.

Group 1: The control group received distilled water orally.

Group II: Received an aluminum chloride solution (100 mg/kg) orally for 42 days (Ahmad Rather et al. 2019; Auti & Kulkarni 2019).

Group III: Received BET at doses of 100 mg/kg orally.

Group IV: Received BET at doses of 200 mg/kg orally.

Group V: Received VCO at doses of 1 g/kg orally.

Group VI: Received VCO at doses of 5 g/kg orally.

Group VII: Received combination at a dose (BET 100mg/kg + VCO 1g/kg)

Group VII: Received combination at a dose (BET 200mg/kg + VCO 5 g/kg)

Group IX: Standard group received Donepezil hydrochloride (1 mg/kg) orally

Group III to VIII received drug treatment for one hour after aluminum chloride administration for 42 days. The rats were exposed to behavioral tests 24 hours after administering the last dosage. The rat cortex and hippocampus were separated for subsequent biochemical and molecular study.

8.3. Behavioral Assessment

8.3.1. Morris Water Maze (MWM)

MWM was performed as per the earlier method described with slight modifications. The MWM test is used to assess spatial memory tasks in rodents (Singh et al., 2018)**.**

8.3.2. Elevated Plus Maze (EPM)

EPM test is used to assess anxiety in rodents and was performed as per the earlier method described (Sharma and Kulkarni, 1992a)

8.3.3. Passive Avoidance Test (PA)

PA test is used to assess rodent memory retention deficits and was performed as per the earlier method described (Barkur and Bairy, 2015)**.**

8.4. Biochemical Parameters

8.4.1. Collection of Brain Tissues

The Hippocampus and cortex parts of the brain were excised. A probe homogenizer (Polytron PT 2500E, Kinematica, Switzerland) was used to homogenize 100 mg of tissue in 0.5 ml phosphate buffer (0.1 M, pH 7.4). The tubes were put on ice to eliminate temperature fluctuations and then

stored at -80^0C. Different fractions of tissue homogenates were produced and processed to yield post-nuclear and post-mitochondrial fractions.

8.4.2. Determination of acetylcholinesterase activity

The AmpliteTM Colorimetric Acetylcholinesterase assay Kit was used to measure AChE activity according to the manufacturer's instructions. The reaction is initiated with the presence of the substrate acetylthiocholine, and the hydrolysis and production of the DTNB dianion were measured at 412 nm within 10 minutes using a microplate reader (ELLMAN et al., 1961; Richetti et al., 2011)

8.4.3. Determination of the oxidative stress parameters

Malondialdehyde (MDA) and reduced glutathione (GSH) assay were measured in the whole homogenate (Ellman, 1959; Ohkawa et al., 1979b). In addition, to extract post-nuclear supernatant (PNS), one fraction was centrifuged at 2,500 rpm for 20 minutes at 4°C. The activity of catalase was determined using this supernatant. (Lück, 1965). Also, a superoxide dismutase (SOD) test was performed with PMS (Paoletti et al., 1986b). The PMS was obtained by centrifuging homogenate at 10,000 rpm for 20 min at 4°C (Suryavanshi et al., 2021)**.**

8.4.4. Interleukins 1β

Per the manufacturer's instructions, the IL-1β level was determined using a commercially available ELISA kit (Krishgen Biosystems, India). Absorbance was read at 450nm within 10-15 min.

8.4.5. Protein determination

The Bradford method measured the protein for the biochemical studies by considering bovine serum albumin as the standard. It is performed using a protein estimation kit. (HI Media Laboratories, India)

8.4.6. Histopathological studies

A histopathology study was performed on brain samples preserved in 10% neutral buffered formalin (NBF). It was embedded in paraffin blocks. A microtome (Thermo Scientific, HM 325) was used to take thin transverse sections (3-5µm thickness) of the hippocampus and cortex area. Hematoxylin, eosin (H&E), and Congo red dye were used to stain the sections, which were then

analyzed under a digital microscope (Nikon, Eclipse, E 100). The sections were observed at a magnification of 400X. The prevalence of neurodegeneration and amyloid beta accumulation was assessed in the brain.

8.5. Statistical Analysis

The data were analyzed using GraphPad PrismV 8.0 for Windows to compare the treatment and control groups. Behavioral parameters were assessed through two-way ANOVA followed by Bonferroni's test to determine significance levels within and between the groups.

9. Effect of Betanin Along with Virgin coconut oil in LPS induced AD in C57BL Mice

9.1. Animals:

Female C57 mice (20-25 g) were procured from Tata Memorial Centre, ACTREC, Kharghar, Navi Mumbai, India. The animals were subjected to a 12-hour light and 12-hour dark cycle, maintained at a temperature of 25°C and relative humidity ranging from 40% to 60%. Prior to the experiment, the animals went through a seven-day period of acclimatization. Animals had access to a standard pellet diet and water ad libitum. The study protocol was approved through Institutional Animal Ethics Committee (approval no. CPCSEA/IAEC/P-29/2020, dated 26th December 2020).

9.2. Experimental Design

Seventy-two female C57 mice (4–5 weeks old) weighing 20-25 g were randomly divided into nine groups, with eight mice in each group. Mice were injected intraperitoneally with 250 µg/kg of Lipopolysaccharide (LPS) or sterile saline (0.9% NaCl) once daily for seven days after the drug treatment. The drug was administered orally to mice for 14 days (once a day).

Sr No.	Group	No. of animals
1	Control	8
2	LPS (250 µg/kg)	8
3	BET (200 mg/kg) + LPS (250 µg/kg)	8
4	BET (400 mg/kg) + LPS (250 µg/kg)	8
5	VCO (5g/kg) + LPS (250 µg/kg)	8
6	VCO (10g/kg) + LPS (250 µg/kg)	8

7	BET (200 mg/kg) + VCO (5g/kg) + LPS (250 µg/kg)	8
8	BET (400 mg/kg) + VCO (10g/kg) + LPS (250 µg/kg)	8
9	Donepezil (2mg/kg) + LPS (250 µg/kg)	8

Table No.1: Grouping of the animals.

9.3. Behavioral Assessment

9.3.1. Morris Water Maze (MWM)

After two weeks of drug administration, the MWM test evaluated the mice's learning and memory abilities in all groups (Liu et al., 2019).

9.3.2. Elevated Plus Maze (EPM)

The procedure, technique, and endpoint for testing learning and memory were followed per the earlier parameters (Sharma and Kulkarni, 1992b).

9.3.3. Locomotor activity

Locomotor activity was performed on day 15^{th}. Each animal was kept in a digital actophotometer and observed for 5min, the apparatus equipped with infrared light-sensitive photocells (Nade et al., 2015)

9.4. Biochemical Parameters Assay

9.4.1. Collection of Brain Tissues

Following the MWM experiment, the female C57 mice were euthanized and their brains were extracted. Tissue homogenates were created and processed to produce post-nuclear and post-mitochondrial fractions..

9.4.2. Determination of acetylcholinesterase activity

The Amplite^{TM} Colorimetric Acetylcholinesterase assay Kit measured AchE activity according to the manufacturer's instructions. The reaction was initiated with the presence of the substrate acetylthiocholine, and the hydrolysis and production of the DTNB dianion were measured at 412 nm within 10 minutes using a microplate reader.

9.4.3. Determination of the oxidative stress parameters

The whole homogenate measured the MDA and GSH assay. In addition, to extract PNS, one fraction was centrifuged at 2,500 rpm for 20 minutes at 4°C. The activity of catalase was determined using this supernatant. Also, a SOD test was performed with PMS. The PMS was obtained by centrifuging homogenate at 10,000 rpm for 20 min at 4°C (Auti and Kulkarni, 2019).

9.4.4. IL-1β determination by ELISA kit

IL-1β was determined using a commercially available Mouse IL-1beta GENLISA™ ELISA (Krishgen Biosystems, India) per the manufacturer's instructions. Absorbance was read at 450nm within 10-15 min. The results are as picograms (pg) of cytokine/mg of tissue.

9.4.5. NLRP3 determination by ELISA kit

Per the manufacturer's instructions, NLRP3 was determined using a commercially available Mouse Nod Like Receptor Pyrin-3, NLRP3 GENLISA™ ELISA (Krishgen Biosystems, India). Absorbance was read at 450nm within 10-15 min. The results are exhibited as nanograms (ng) /mg of tissue.

9.4.6. Histopathological studies

Brain tissues were stored in 10% neutral buffered formalin (NBF) and used for histopathology study. It was embedded in paraffin blocks, and thin transverse sections of 3-5 µ thickness were taken using a microtome (Thermo scientific, HM 325) of the hippocampus and cortex region. The sections were stained with hematoxylin and eosin (H&E) and examined under a digital microscope (Nikon, Eclipse, E 100). The observations of the sections were carried out at 400X. Morphometric analysis was carried out to evaluate the extent of neurodegeneration.

9.5. Statistical Analysis

The data regarding behavior was subject to analysis through GraphPad Prism 8.0 for Windows. Our team utilized a one-way ANOVA to compare the treatment and control groups, implementing a significance diffrence of $p<0.005$ for evaluating both within-group and between-group variances. To assess differences among the groups, the Dunnet test was employed.

10. Results:

10.1. Characterization of Betanin by LCMS

The betanin HPLC chromatograms were measured at 534 nm (betacyanins) and 484nm for (betaxanthin). We observed the presence of betanin and isobetanin that showed retention times of 5.8 and 7.3 min at 535nm, whereas, at 484 nm, betaxanthins showed a retention time of 3.4min.

10.2. Characterization of virgin coconut oil by GCMS

The fatty acid profile of virgin coconut oil was determined using GC–MS/FID. Their mass fragmentation analysis confirmed the identity of the main constituents. The principal fatty acid found in oil were: Caproic acid (0.6%), Caprylic acid (8.17%), Lauric acid (48.31%), and Myristic acid (19.82%).

10.3. Acute toxicity study of the combination

In an experimental study, a cohort of rats was subjected to oral administrations of BET in dosages of 1000 and 2000 mg/kg and VCO in quantities of 5 and 10 g/kg. The study results indicated that the rats did not display any adverse signs of toxicity or mortality, suggesting that the combined dosages of BET and VCO were not harmful at the prescribed dose. Furthermore, corroborating evidence highlights that the administration of BET and VCO produced no deleterious effects.

10.4. Betanin mitigates scopolamine-induced cognitive impairment by restoring cholinergic function, boosting brain antioxidative status, and increasing BDNF levels in the zebrafish model.

Based on the study's results, it was found that the zebrafish did not exhibit any behavioral abnormalities or signs of distress after being exposed to different doses of BET, ranging from 50 μL/L to 200 μL/L. It indicated that BET can be safely administered to zebrafish. Conversely, administering scopolamine to the fish resulted in the manifestation of anxiety and cognitive impairments. Also, the specific activities of antioxidant enzymes were significantly reduced SOD ($p < 0.01$), CAT ($p < 0.05$), GSH ($p < 0.00001$), and elevated levels of lipid peroxidation (MDA) ($p < 0.05$). The scopolamine-treated group had substantially increased AChE-specific activity($p < 0.0001$) and decreased BDNF levels in the brain of fish ($p < 0.01$) in contrast to the control group. However,Betanin (50 and 100 mg/L) showed cognitive enhancing, anti-acetylcholinesterase ($p <$

0.0001), antioxidant, and increased BDNF level($p < 0.01$ for 50 mg/L, $p < 0.001$for 100mg/L) in the scopolamine-induced zebrafish model.

10.5. The effects of Betanin and Virgin Coconut oil in aluminum chloride triggered an AD model in rats via the NLRP3 inflammasome pathway.

BET (1000 and 2000 mg/kg) and VCO (5 and 10 g/kg) orally to a group of rats. The aluminum chloride-treated group had markedly increased escape latency (EL) in the MWM test ($p < 0.0001$), transfer latency in the EPM ($p < 0.0001$), and retention latency in the PA test ($p < 0.0001$) compared to the control group. The combination significantly decreases EL ($p < 0.01$for 100mg/kg +1g/kg; $p < 0.0001$ for 200mg/kg + 5g/kg), TL ($p < 0.0001$ for BET 100mg/kg + VCO 1g/kg,$p < 0.0001$ for BET 200mg/kg + VCO 5g/kg), and RL ($p < 0.0001$ for BET 100mg/kg + VCO 1g/kg; $p < 0.01$ for BET 200mg/kg + VCO 5 g/kg) compared to The aluminum chloride-treated group.

The combination (BET 100 mg/kg + VCO 1 g/kg and BET 200 mg/kg + VCO 5 g/kg) subsided the acetylcholinesterase activity more effectively compared to the aluminum chloride group (in the hippocampus $p < 0.0001$, in the cortex $p < 0.05$).

Combination treatment increased GSH activity ($p < 0.05$ for BET 200 mg/kg + VCO 5g), decreased MDA activity($p < 0.05$ for BET 100 mg/kg + VCO 1g/kg and BET 200 mg/kg + VCO 5g/kg)., increased CAT activity ($p < 0.05$ for BET 100 mg/kg + VCO 1g/kg) in the hippocampus. Also, in the cortex, they augmented SOD activity($p < 0.05$ for BET 100 mg/kg + VCO 1 g/kg and BET 200 mg/kg + VCO 5g), MDA activity ($p < 0.0001$ for BET 100 mg/kg + VCO 1g/kg and BET 200 mg/kg + VCO 5g/kg) increased GSH activity ($p < 0.01$ for BET 200 mg/kg + VCO 5g) by subsiding oxidative stress compared to an aluminum chloride group.

In the hippocampus, the combination significantly reduced IL-1β ($p < 0.05$ for BET 100mg/kg + VCO 1g/kg and BET 200mg/kg + VCO 5g/kg) and NLRP3 level ($p < 0.05$ for BET 200mg/kg + VCO 5g/kg and $p < 0.01$ for BET 100mg/kg + VCO 1g/kg).In the cortex, the treatment with combination significantly reduced IL-1β level ($p < 0.01$ for BET 100mg/kg + VCO 1g/kg and $p < 0.001$ for BET 200mg/kg + VCO 5 g/kg) and NLRP3 level ($p < 0.05$ for BET 200 mg/kg + VCO 5 g/kg).

The administered treatment exhibited a notable reduction in the extent of damage and mortality of brain cells in both the hippocampus and cortex. In addition, it significantly mitigated the intensity

of anomalous cellular activity, plaque formation, and cell death rate in the CA3 region of the hippocampus.

10.6. Betanin and Virgin coconut oil ameliorate neuroinflammation induced by lipopolysaccharides in a C57BL mouse by inhibiting the NLRP3 inflammasome pathway.

LPS treated group showed a significant increase in escape latency compared to the normal control group ($p < 0.05$). When compared to the control group, the LPS group showed a significant deficit in cognition ($p < 0.0001$), whereas this result was altered when BET and VCO were used (BET 400 mg/kg,$p < 0.05$, VCO 10g/kg $p < 0.01$). In EPM, the treatment showed a significant decrease in transfer latency compared to the LPS-treated group. $F (8, 63) = 5.308$ $P< 0.0001$. LPS treatment caused a significant increase in acetylcholinesterase level in the brain of a mouse ($p < 0.01$) in contrast to the control group. However, treatment significantly decreases the level of acetylcholinesterase compared to LPS-treated animals. $F (8, 45) = 3.487$, $P=0.0033$.

The treatment group significantly improved the oxidative status compared to LPS treated group. SOD $F (8, 45) = 7.644$, $P<0.0001$, CAT $F (8, 45) = 2.510$, $P=0.0239$, the total content of reduced GSH. $F (8, 43) = 10.87$, $P < 0.0001$, and elevated levels of lipid peroxidation (MDA). $F (8, 45) = 4$, $P=0.0006$.

LPS treatment caused a significant increase in IL-1β and NLRP3 levels in the brain of a mouse. Treatment of betanin and VCO significantly decreases the level of IL-1β ($F (8, 43) = 4$, $P=0.0002$) and NLRP3 ($F (8, 44) = 4$ $P=0.0007$) compared to LPS-treated animals.

The group receiving treatment with BET 200, 400 mg/kg, and VCO 2 g/kg and 10 g/kg showed a decrease in the extent of damage to brain cells in the hippocampus and cortex. It was observed through H and E staining, which revealed reduced neuronal degeneration and necrosis.

11. Conclusion:

According to our research, applying BET in 50 and 100 mg/L concentrations has positively impacted cognitive function in zebrafish models with scopolamine-induced impairments. Furthermore, BET has also been found to reduce acetylcholinesterase activity and increase levels of the antioxidant BDNF in these models. These findings offer promising evidence that betanin could potentially be developed as a treatment option for AD.

In our research, we found that a combination of VCO and BET (with doses of BET at 100mg/kg +VCO 1 g/Kg and BET at 200mg/kg +VCO 5g/Kg) can protect the brain cells of rats caused by $AlCl_3$. This combination has antioxidant and anti-inflammatory properties that improve cognitive function and reduce AChE activity, NLRP3, and IL-1β levels in rats. Our findings suggest that this combination therapy can be a novel treatment option for AD by regulating the NLRP3 inflammasome pathway.

In the LPS-induced C57BL mice model, betanin, along with virgin coconut oil, was found to inhibit AChE activity, lowered oxidative stress, and control neuroinflammation in LPS-induced cognitive impairment in C57BL mice suggesting the neuroprotective effect of the combination in managing neuroinflammation-induced AD.

12. Publication Details:

Sr. No.	Details of publications	Impact factor	Status
1	Thawkar, B.S., Kaur, G., 2018. Inhibitors of NF-κB and P2X7/NLRP3/Caspase 1 pathway in microglia: Novel therapeutic opportunities in neuroinflammation induced early-stage Alzheimer's disease. J. Neuroimmunol. 326, 62–74.	3.478	Published
2	Thawkar, B.S., Kaur, G., 2021. Zebrafish as a Promising Tool for Modeling Neurotoxin Induced Alzheimer's Disease. Neurotox. Res. https://doi.org/10.1007/s12640-021-00343-z	3.911	Published
3	Thawkar, B.S., Kaur, G., Betanin mitigates scopolamine-induced cognitive impairment by restoring cholinergic function, boosting brain antioxidative status, and increasing BDNF level in the zebrafish model. Fish Physiology and Biochemistry	2.794	Published
4	Thawkar, B.S., Kaur, G., Banerjee M., Alzheimer's disease preliminary screening in zebrafish, integrating behavioural models and molecular markers. Handbook of Animal Models in Neurological Disease. Academic Press- Elsevier.	Elsevier	Published
5	Thawkar, B.S., Kaur, G., The dietary combination's effects of Betanin and Virgin Coconut oil in aluminum chloride triggered an Alzheimer's disease model in rats via the NLRP3 inflammasome pathway—Journal of Traditional and complementary medicine.	Elsevier 4.2	Under review

6	Thawkar, B.S., Kaur, G., Betanin with virgin coconut oil attenuates lipopolysaccharide-induced cognitive impairment via modulation of NLRP3/IL1β in C57BL mice—Inflammation.	Springer	Under review

Presentation:

Baban S Thawkar, Ginpreet Kaur, Betanin ameliorates scopolamine-induced cognitive impairment by restoring cholinergic function, enhancing brain antioxidative state, and elevating BDNF levels in the zebrafish model. 10th International Conference & Exhibition (SPER 2021) organized by SPER and GUNI-SKPCPER on 08 - 09th October 2021 at Ganpat University, Mehsana (Gujarat), India.

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